

## Baicalein protects against the development of angiotensin II-induced abdominal aortic aneurysms by blocking JNK and p38 MAPK signaling

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An abdominal aortic aneurysm (AAA) is a permanent, localized dilatation of the abdominal aorta. In western countries, the morbidity of AAA is approximately 8%. Currently, pharmacotherapies for AAA are limited. Here, we demonstrate that baicalein (BAI), the main component of the Chinese traditional drug “Huang Qin”, attenuates the incidence and severity of AAA in *Apoe*<sup>−/−</sup> mice infused with angiotensin II (AngII). Mechanically, BAI treatment decreases AngII-induced reactive oxygen species (ROS) production in the aortic wall. Moreover, BAI inhibits inflammatory cell accumulation in the aortas of mice infused with AngII. It also inhibits AngII-induced activation of matrix metalloproteinase 2 (MMP-2) and MMP-9 to maintain elastin content *in vivo*. In addition, it blocks AngII cascade by downregulating angiotensin type 1 receptor (AT1R) and inhibiting mitogen-activated protein kinases (MAPKs). Taken together, our findings show that BAI is an effective agent for AAA prevention.

**baicalein, abdominal aortic aneurysm, oxidative stress, vascular inflammation, extracellular matrix degradation, AT1R, MAPKs**

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### INTRODUCTION

An abdominal aortic aneurysm (AAA) is defined as a permanent, localized dilatation of the abdominal aorta to 1.5-fold more than the normal diameter. In western countries, the morbidity of AAA is approximately 8%. Owing to the lack of typical syndrome, the mortality rate associated with aortic rupture exceeds 80% (Nordon et al., 2011; Sakalihasan et al., 2005). Currently, AAA therapies mainly include pharmacotherapy and surgical approaches (open repair and endovascular aneurysm repair) (Brewster et al.,

2003). The chief function of pharmacotherapy, which controls the risk factors, is to indirectly attenuate aneurysm development and aorta rupture by regulating blood pressure, serum lipid level, and blood coagulation (Bergoeing et al., 2006; Miyake and Morishita, 2009). Therefore, developing new drugs that target the mechanism of AAA development is of great significance to AAA prevention and treatment.

The involved pathophysiological processes mainly include oxidative stress, vascular inflammation, and extracellular matrix degradation (Lopez-Candales et al., 1997; Thompson et al., 1997, 2002). *Apoe*<sup>−/−</sup> mice infused with angiotensin II (AngII) is the most commonly employed animal model to study the pathogenesis of AAA since it

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mimics almost all the clinical characteristics of human AAA (Daugherty and Cassis, 2004; Weiss et al., 2001). Notably, AngII cascade is upregulated in human AAA samples (Annambhotla et al., 2008; Golledge and Powell, 2007). Baicalein (BAI), which is involved in oxidative stress, inflammation, tumorigenesis, and angiogenesis, is the main component of the Chinese traditional drug “Huang Qin”, (Gao et al., 1999). It has been reported that BAI remarkably retards ischemia/reperfusion-induced myocardium injury (Liu et al., 2013; Wang et al., 2013). However, its exact mechanism remains elusive and its role in AAA development has not been elucidated.

In the present study, we examined the role of BAI in AAA induced by AngII treatment in *Apoe*<sup>-/-</sup> mice. From the view of oxidative stress, vascular inflammation, and extracellular matrix degradation, the relevant mechanisms were studied. The AngII cascade activation was also evaluated under BAI treatment. We believe that our findings may provide a new drug for AAA prevention and treatment.

## RESULTS

### BAI decreases AAA incidence *in vivo*

BAI did not alter the gross morphology of the aortas in *Apoe*<sup>-/-</sup> mice (Figure 1A). Consistent with previous findings (Satoh et al., 2009; Wang et al., 2012), the incidence of

AAA in *Apoe*<sup>-/-</sup> mice treated with vehicle after AngII infusion for 4 weeks was 88% in our study. In contrast, only 47% of BAI-treated *Apoe*<sup>-/-</sup> mice developed AAA after AngII treatment (Figure 1B). No AAAs were detected in saline-infused mice.

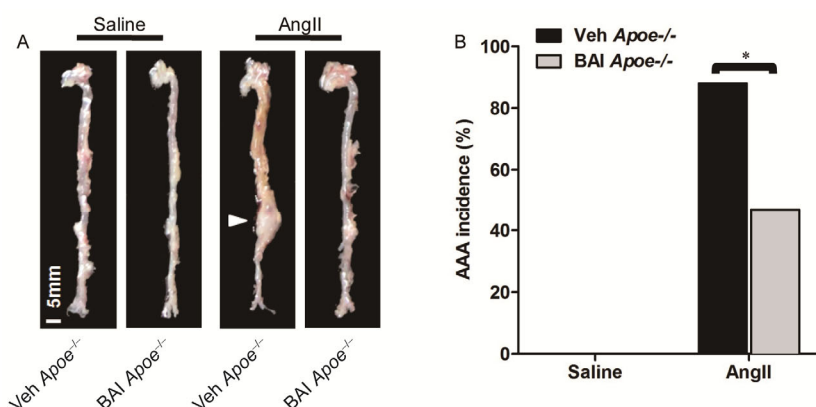
### BAI attenuated AAA severity (the ratio of aorta weight to body weight and maximal abdominal aortic diameter) *in vivo*

The maximal abdominal aortic diameter and the total aortic weight significantly increased after AngII infusion for 4 weeks in all groups compared to that in saline-infused mice. Compared to AngII-infused mice treated with vehicle, the BAI-treated mice had decreased maximal abdominal aortic diameter and total aortic weight (Figure 2A–C). As shown in Tables 1 and 2, SBP, HR, and serum lipid levels did not differ between *Apoe*<sup>-/-</sup> mice treated with BAI and those receiving vehicle after AngII infusion for 4 weeks.

Taken together, BAI represses AngII-induced AAA *in vivo*, but it did not affect hemodynamic and lipid metabolic indices.

### BAI treatment attenuated AngII-induced ROS production *in vivo*

Since oxidative stress is critical in AAA formation (Satoh et al., 2009; Thomas et al., 2006), we first determined the oxi-



**Figure 1** (color online) Baicalein (BAI) treatment represses abdominal aortic aneurysms (AAA) formation in *Apoe*<sup>-/-</sup> mice infused with angiotensin II (AngII). Mice were pre-treated with vehicle (Veh) or BAI (30 mg kg<sup>-1</sup> day<sup>-1</sup>) via intraperitoneal injection for 1 week before infusion with saline or AngII for 4 weeks. Treatment with vehicle or BAI (30 mg kg<sup>-1</sup> day<sup>-1</sup>) via intraperitoneal injection was performed at the same time-point every day during the saline or AngII infusion. A, Representative photographs showing macroscopic features of aneurysms induced by AngII following treatment with vehicle or BAI. The arrows show typical AAAs (scale bars, 5 mm). B, The incidence of AngII-induced AAA in vehicle-treated *Apoe*<sup>-/-</sup> mice (*n*=17) relative to BAI-treated *Apoe*<sup>-/-</sup> mice (*n*=17). AAAs were not detected in saline-infused mice (*n*=6 per group). \*, *P*<0.05.

**Table 1** Blood pressure and heart rate in mice<sup>a)</sup>

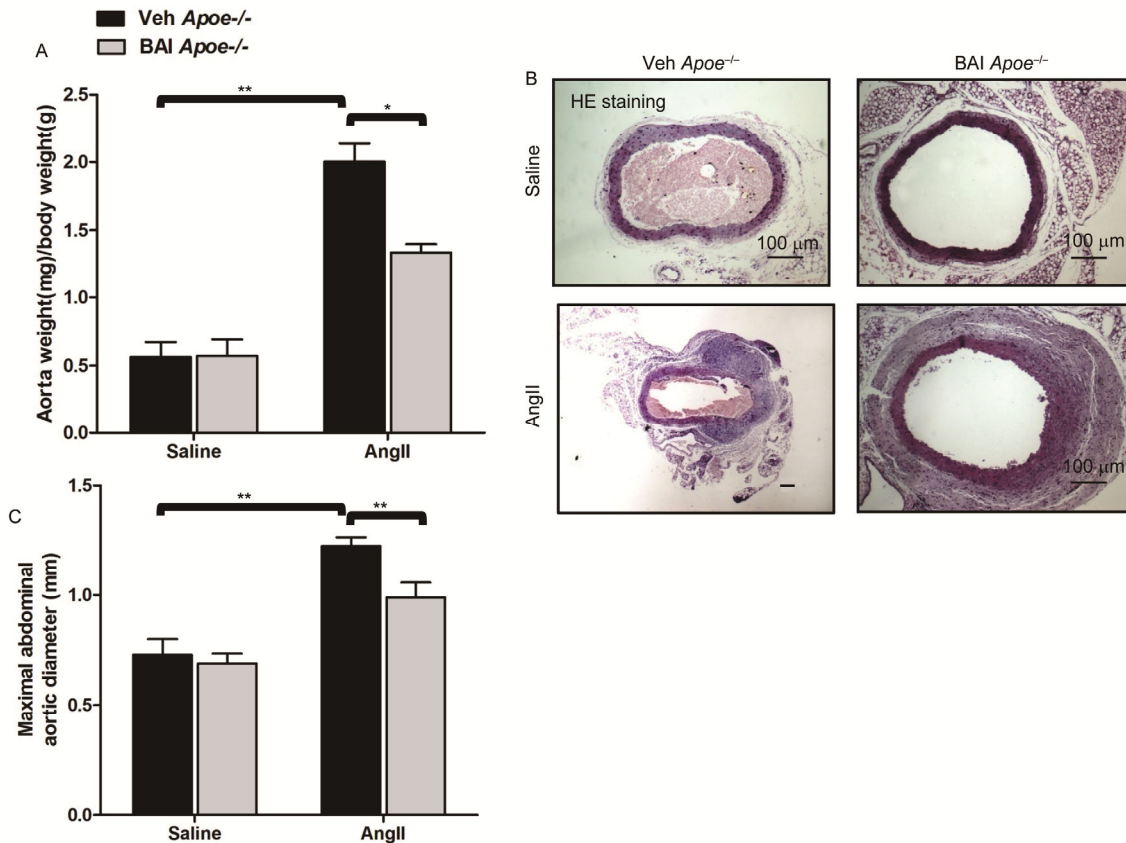
Groups	SBP (Baseline, mmHg)	SBP (AngII, mmHg)	HR (Baseline, beats min <sup>-1</sup> )	HR (AngII, beats min <sup>-1</sup> )
Veh <i>Apoe</i> <sup>-/-</sup>	98.6±5.8	129.3±3.8*	553.8±13.6	524.6±16.0
BAI <i>Apoe</i> <sup>-/-</sup>	97.4±6.4	126.6±5.7*	538.3±18.6	535.7±22.4

a) *n*=5 in baseline group; *n*=10 in AngII group; \*, *P*<0.05 compared to baseline; data are mean±SE.

**Table 2** Serum lipid levels in AngII-infused mice<sup>a)</sup>

Groups (AngII-infused)	Cholesterol (mmol L <sup>-1</sup> )	Triglyceride (mmol L <sup>-1</sup> )	HDL-C (mmol L <sup>-1</sup> )	LDL-C (mmol L <sup>-1</sup> )
Veh <i>Apoe</i> <sup>-/-</sup>	16.36±1.40	2.17±0.15	0.85±0.09	2.61±0.26
BAI <i>Apoe</i> <sup>-/-</sup>	15.73±0.98	2.43±0.41	0.91±0.04	2.71±0.27

a) Results are expressed as mean±SE, *n*=10 in each group.



**Figure 2** Baicalein (BAI) treatment attenuates abdominal aorta dilatation in *Apoe*<sup>-/-</sup> mice infused with angiotensin II (AngII). A and C, The ratio of the aorta weight to body weight (A) and maximal abdominal aortic diameter (C) in saline- and AngII-infused mice following treatment with vehicle or BAI (*n*=5 in each group of *Apoe*<sup>-/-</sup> mice infused with saline; *n*=14 for AngII-infused *Apoe*<sup>-/-</sup> mice following treatment with vehicle; *n*=15 for AngII-infused *Apoe*<sup>-/-</sup> mice following treatment with BAI). B, Representative H&E staining of suprarenal aortas from saline- and AngII-infused mice treated with vehicle or BAI. Scale bars, 100 μm. Error bars in A and C represent the mean±SE. \*, *P*<0.05; \*\*, *P*<0.01.

ductive stress-related parameters *in vivo*. We analyzed vascular ROS production by quantification of dihydroethidine hydrochloride (DHE) staining. DHE-positive staining was rarely detected in the suprarenal aortic wall of saline-infused mice. AngII infusion markedly increased the level of ROS production in *Apoe*<sup>-/-</sup> mice. However, densitometric analysis of DHE staining showed reduced staining in the suprarenal aortic wall of BAI-treated *Apoe*<sup>-/-</sup> mice (Figure 3A and B). Collectively, BAI ameliorates AngII-induced ROS *in vivo*.

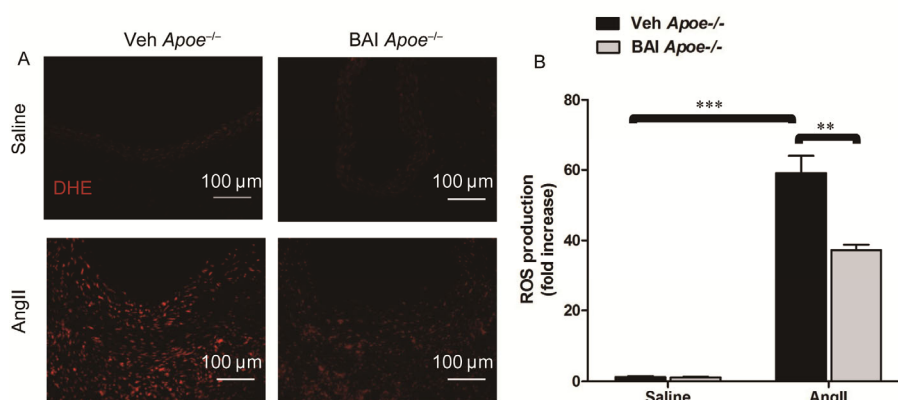
#### BAI treatment decreased AngII-induced vascular inflammation *in vivo*

Vascular inflammation is implicated in AAA development, which is correlated with ROS production and MMP activa-

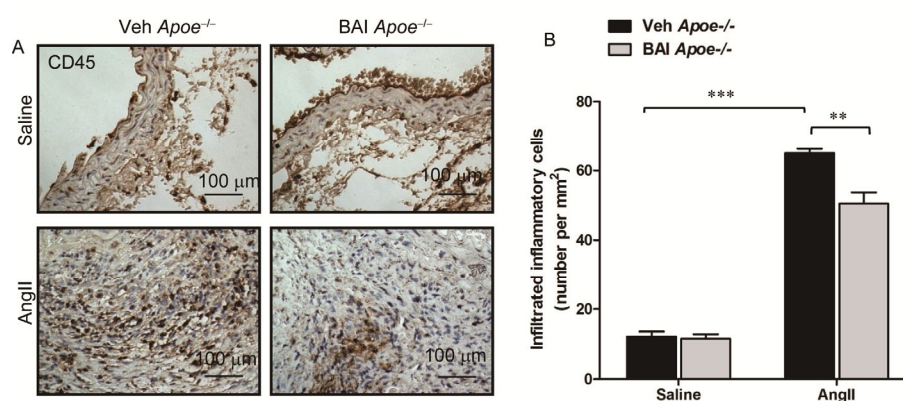
tion (Hellenthal et al., 2009b; Thompson et al., 2002). Few inflammatory cells were found in the suprarenal aortic wall of saline-infused mice. After AngII infusion, a large number of inflammatory cells, as determined by CD45 immunostaining, accumulated in the aortic wall especially in the adventitia of *Apoe*<sup>-/-</sup> mice. However, the number of inflammatory cells was remarkably lower in the suprarenal aortic wall of AngII-infused *Apoe*<sup>-/-</sup> mice after BAI treatment (Figure 4A and B), than that after vehicle treatment.

#### BAI treatment inhibited AngII-induced MMP-2 and MMP-9 activation to maintain elastin content *in vivo*

Vascular smooth muscle cell (VSMC)-derived MMP-2 and macrophage-derived MMP-9 are considered as key factors in extracellular matrix degradation that is crucial for AAA



**Figure 3** Baicalein (BAI) treatment inhibits angiotensin II (AngII)-induced reactive oxygen species (ROS) production *in vivo*. A, Representative DHE staining of suprarenal aortic wall of saline- and AngII-infused *Apoe*<sup>-/-</sup> mice treated with vehicle or BAI (scale bars, 100  $\mu$ m). B, Densitometric analysis of DHE staining of suprarenal aortic wall of saline- and AngII-infused *Apoe*<sup>-/-</sup> mice treated with vehicle or BAI.  $n=6$  per group. \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$ . The error bars in B are mean $\pm$ SE.



**Figure 4** Baicalein (BAI) treatment inhibits angiotensin II (AngII)-induced vascular inflammation *in vivo*. A, Representative immunostaining with CD45 of the suprarenal aortic wall of *Apoe*<sup>-/-</sup> mice infused with saline or AngII following treatment with vehicle or BAI (scale bars, 100  $\mu$ m). B, The number of CD45-positive cells accumulating in the suprarenal aortic wall of saline- ( $n=5$  per group) and AngII-infused mice ( $n=7$  per group) following treatment with vehicle or BAI. \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$ . Error bars in B represent the mean $\pm$ SE.

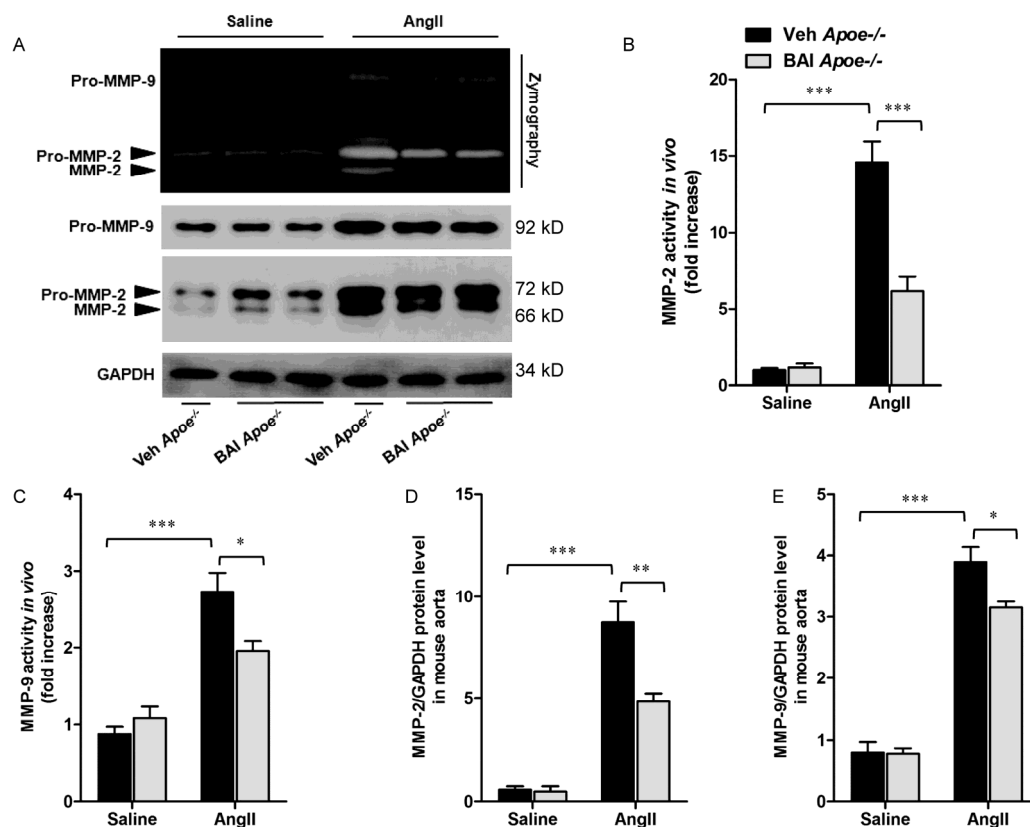
development and aortic rupture (Longo et al., 2002). First, we examined the activity of MMP-2 and MMP-9 in aorta homogenates by gelatin zymography. AngII infusion remarkably induced MMP-2 activation (14.6-fold increase on an average) and MMP-9 activation (2.7-fold increase on an average) in *Apoe*<sup>-/-</sup> mice; a lower degree of activation was observed in BAI-treated *Apoe*<sup>-/-</sup> mice (MMP-2: 6.1-fold increase on an average; MMP-9: 1.9-fold increase on an average; Figure 5A–C). The expression of MMP-2 and MMP-9 markedly increased in AngII-infused *Apoe*<sup>-/-</sup> mice. In contrast to the results obtained for vehicle groups, BAI treatment reduced MMP-2 and MMP-9 protein level after AngII treatment (Figure 5A, D and E).

Next, medial elastin content was measured to detect extracellular matrix degradation. A marked decrease in aortic elastic fiber content was detected after AngII infusion for 4 weeks relative to saline-infused mice. However, the elastin fiber content in the suprarenal aortas of BAI-treated mice

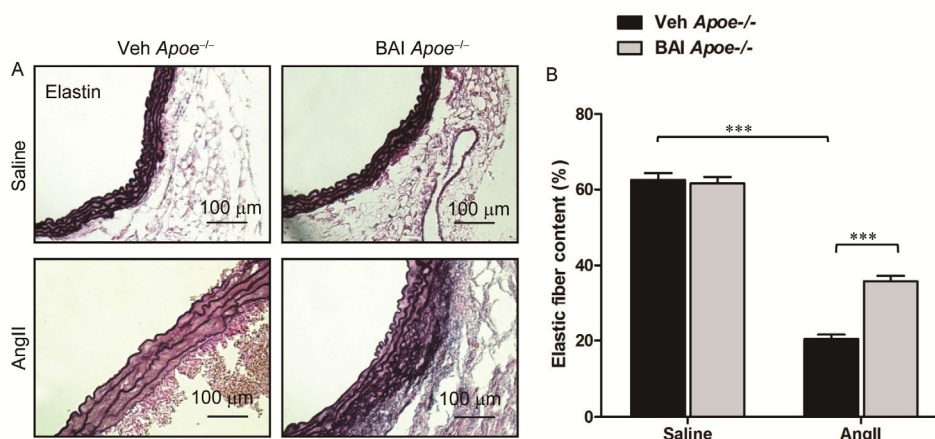
after AngII infusion was significantly higher than that in vehicle-treated mice (Figure 6A and B). Taken together, these *in vivo* data suggest that AngII-induced MMP activation and extracellular matrix degradation are inhibited by BAI.

#### BAI treatment blocked p38-mitogen activated protein kinase (MAPK) and JNK signaling pathways by down-regulating AT1R *in vivo*

The physiological and pathological effects of AngII are mainly mediated by its receptors, AT1R and AT2R (Mehta and Griendling, 2007). Thus, we examined whether BAI-treatment could influence the level of AngII receptors in mouse aorta. The data in Figure 7A–C shows that the protein level of AT1R was repressed after BAI treatment in the aortas of both saline- or AngII-infused *Apoe*<sup>-/-</sup> mice. However, the AT2R expression was not affected by BAI administration. Next, we determined the downstream sig-



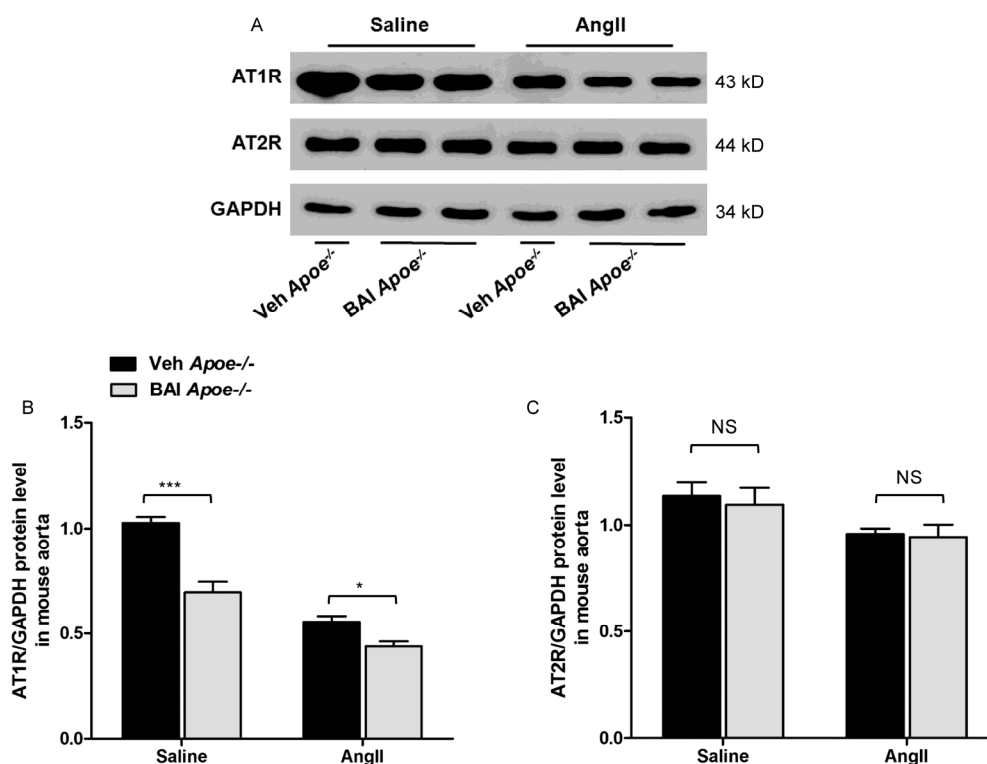
**Figure 5** Baicalein (BAI) treatment decreases angiotensin II (AngII)-induced matrix metalloproteinase (MMP)-2 and MMP-9 activation *in vivo*. A, Representative gelatin zymography and western blots of MMP-2 and MMP-9 *in vivo*. B and C, Densitometric analysis of MMP-2 (B) and MMP-9 (C) activity in aorta homogenates. D and E, Densitometric analysis of MMP-2 (D) and MMP-9 (E) expression in aorta homogenates. Aorta homogenates were obtained from *ApoE*<sup>-/-</sup> mice infused with saline or AngII for 4 weeks following treatment with vehicle or BAI. (*n*=8 in each group of *ApoE*<sup>-/-</sup> mice infused with AngII; *n*=5 in each group of mice infused with saline). \*, *P*<0.05; \*\*, *P*<0.01; \*\*\*, *P*<0.001. Error bars in B–E represent the mean±SE.



**Figure 6** Baicalein (BAI) treatment ameliorates angiotensin II (AngII)-induced elastin degradation *in vivo*. A, Representative staining with elastin of suprarenal aortas from saline- and AngII-infused mice treated with vehicle or BAI. Scale bars, 100  $\mu$ m. B, Quantitative analysis of elastin fiber content by quantification of the ratio of elastic fiber area to the suprarenal aortic media area in suprarenal aortas from saline and AngII-infused mice treated with vehicle or BAI. *n*=5 in each group of *ApoE*<sup>-/-</sup> mice infused with saline. *n*=7 in the group of AngII-infused *ApoE*<sup>-/-</sup> mice. \*\*\*, *P*<0.001. The error bars in B are mean±SE.

naling cascades, MAPK activation, in the aortas of saline- or AngII-infused *ApoE*<sup>-/-</sup> mice. After AngII infusion for 4 weeks, p38-MAPK, Erk, and JNK signaling were upregu-

lated. In BAI-treated groups following AngII-infusion, p38-MAPK and JNK signaling but not Erk activation were attenuated, compared to vehicle-treated groups (Figure



**Figure 7** Baicalein (BAI) reduces angiotensin type-1 receptor (AT1R) but not AT2R protein expression *in vivo*. A, Representative western blots of AT1R and AT2R *in vivo*. B and C, Densitometric analysis of AT1R (B) and AT2R (C) expression in aorta homogenates. The data of vehicle-treated mice infused with saline have been normalized as 1. Aorta homogenates were obtained from  $Apoe^{-/-}$  mice infused with saline or AngII for 4 weeks following treatment with vehicle or BAI. ( $n=7$  in each group of  $Apoe^{-/-}$  mice infused with AngII;  $n=5$  in each group of mice infused with saline). \*,  $P<0.05$ ; \*\*\*,  $P<0.001$ . NS represents no significance. Error bars in B and C represent the mean $\pm$ SE.

8A–D). In summary, BAI inhibits AngII cell signaling by downregulating AT1R *in vivo*.

## DISCUSSION

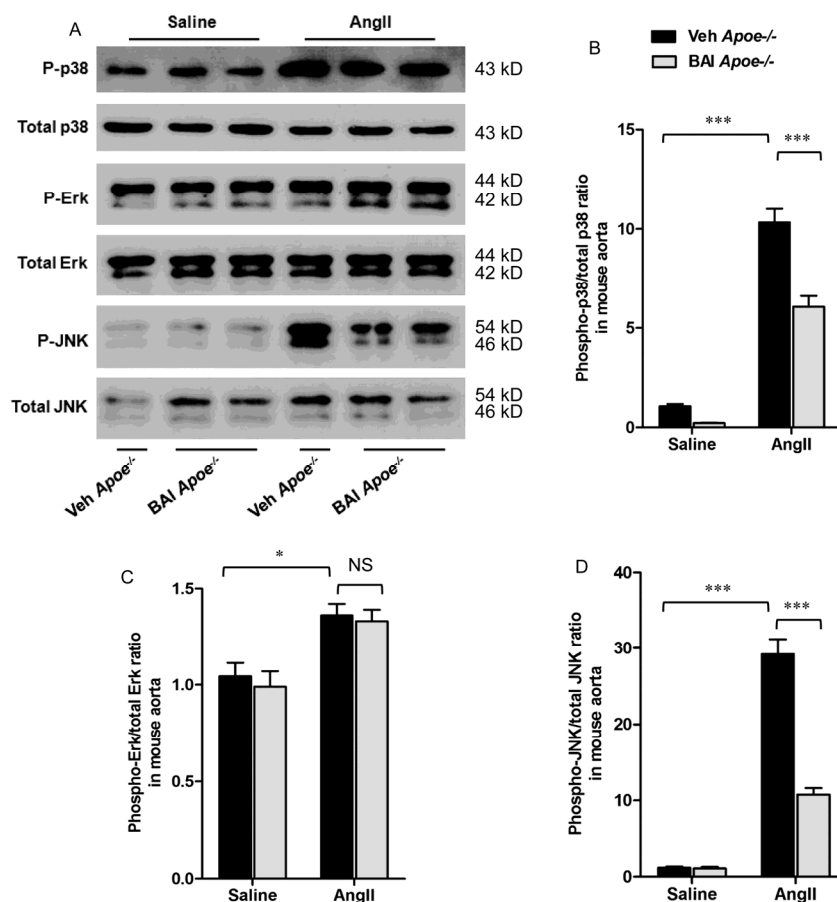
In this study, we showed for the first time that BAI protects against AngII-induced AAA in  $Apoe^{-/-}$  mice. BAI treatment significantly reduces AAA incidence and aorta dilatation *in vivo*. Mechanically, it attenuates AngII-induced oxidative stress, vascular inflammation, and extracellular matrix degradation. Moreover, it inhibits JNK and p38-MAPK signaling by AT1R downregulation (summarized in Figure 9). Our findings suggest a new agent for AAA pharmacotherapy.

Currently, there is no effective pharmacological therapy to prevent or suppress AAA development and progression. Intraperitoneal injection of BAI (30 mg kg<sup>-1</sup> day<sup>-1</sup>) markedly reduced the incidence of AAA in AngII-infused  $Apoe^{-/-}$  mice. More importantly, the severity of AAA (the ratio of aorta weight to body weight and maximal abdominal aortic diameter) decreased in response to BAI treatment in AngII-infused mice, suggesting that BAI protects against not only AAA initiation but also its progression.

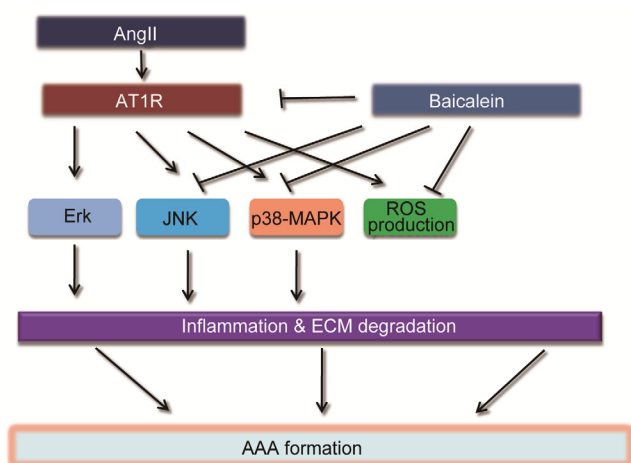
Augmented oxidative stress plays a vital role in AAA

development (Keaney, 2005; Zhang et al., 2003). In the present study, we found that ROS production as examined by DHE staining was inhibited in mouse aortas after BAI treatment. The mechanisms underlying the antioxidant effect of BAI mainly include ROS scavenging and decreased ROS production (Gao et al., 1999; Kang et al., 2012; Shao et al., 1999). Moreover, oxidative stress is closely associated with inflammation and MMP activation (Hellenthal et al., 2009a, b). For example, the expression of monocyte chemotactic protein 1 (MCP-1) and MMP-2 is partly regulated by some redox-sensitive transcriptional factors such as nuclear factor (NF)- $\kappa$ B (Finkel and Holbrook, 2000). Thus, decreased ROS production leads to the inhibition of subsequent inflammation and extracellular matrix degradation. As shown in our figures, inflammatory cell accumulation in aortic wall is attenuated by BAI treatment. According to our data, inflammatory cells are mainly localized in vascular adventitia, indicating that the cells may be from the blood circulation. Previous reported data show that the majority of ROS is secreted by inflammatory cells such as monocytes and macrophages (Thompson et al., 2002). Thus, BAI-induced attenuation of inflammation also results in decreased ROS production. During AAA development, the vascular wall stress increases following extra





**Figure 8** Baicalein (BAI) inhibits p38-mitogen activated protein kinase (MAPK) and JNK but not Erk activation *in vivo*. A, Representative western blots of p38-MAPK, Erk, and JNK *in vivo*. B–D, Densitometric analysis of p38-MAPK (B), Erk (C), and JNK (D) expression in aorta homogenates. The data of vehicle-treated mice infused with saline have been normalized as 1. Aorta homogenates were obtained from *Apoe*<sup>−/−</sup> mice infused with saline or AngII for 4 weeks following treatment with vehicle or BAI. (*n*=7 in each group of *Apoe*<sup>−/−</sup> mice infused with AngII; *n*=5 in each group of mice infused with saline). \*, *P*<0.05; \*\*\*, *P*<0.001. NS represents no significance. Error bars in B–D represent the mean±SE.



**Figure 9** (color online) Baicalein (BAI) plays a critical role in angiotensin II (AngII) cascade inhibition. AngII binds to its receptor angiotensin type-1 receptor (AT1R) to activate mitogen activated protein kinases (MAPKs) and to induce reactive oxygen species (ROS) production, which further increases vascular inflammation and ECM degradation. BAI attenuates AngII-induced oxidative stress, vascular inflammation, and extracellular matrix degradation *in vivo*. Mechanically, BAI inhibits JNK and p38-MAPK signaling by AT1R downregulation.

cellular matrix degradation, which leads to the final aortic rupture (Vorp, 2007). VSMC-derived MMP-2 and macrophage-derived MMP-9 are of great importance in the process of ECM degradation (Galis and Khatri, 2002; Longo et al., 2002). Our data show that both the expression and the activity of MMP-2 and MMP-9 are inhibited by BAI treatment, indicating that the main target of BAI may be VSMCs and macrophages. These effects of BAI result in the preservation of the wavy structure of the elastic lamellae and the elastic content to prevent AAA development and aortic rupture.

The majority of the known effects of AngII are mediated by AT1Rs, which are widely distributed in the lung, kidney, and heart. While the majority of the vasoactive effects of AngII occur via AT1Rs, AT2Rs have been shown to have anti-proliferative and pro-apoptotic effects in VSMCs, mainly by antagonizing AT1Rs (Griendling et al., 1996). Our data show that in saline- and AngII-infused aortas, AT1R but not AT2R is downregulated with BAI treatment, resulting in AngII cascade blockade. For the downstream signaling pathways, we examined MAPK activation *in vivo*,

which is implicated in cellular protein synthesis, gene expression, metabolism, and growth. Interestingly, JNK and p38-MAPK but not Erk pathways were downregulated following BAI treatment. It has been reported that inhibition of JNK pathway prevents AAA development through MMP-2 and MMP-9 suppression (Yoshimura et al., 2005). Our results and previous data indicate that MAPK signaling pathways could be considered novel therapeutic targets of AAA treatment.

In conclusion, BAI treatment in *Apoe*<sup>-/-</sup> mice inhibits AAA formation induced by AngII infusion and is associated with a reduction of oxidative stress, vascular inflammation, and ECM degradation. The effects of BAI are mediated by the inhibition of JNK and p38-MAPK pathways through AT1R downregulation. Our findings represent novel therapeutic agent particularly for prevention of AAA initiation and progression.

## MATERIALS AND METHODS

### Generation of mice

All the animal protocols were approved by the Animal Care and Use Committee at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College (CAMS&PUMC). The *Apoe*<sup>-/-</sup> and C57BL/6J mice were obtained from Peking University (Beijing).

### Blood pressure and serum lipid measurements

Heart rate (HR) and systolic blood pressure (SBP) of the animals were measured using tail-cuff plethysmography (BP-2000 System, Visitech Systems, Apex, USA) as described previously (Li et al., 2011). Blood samples were obtained from the mice before sacrifice. The blood was collected from the abdominal vena cava at sacrifice. Serum cholesterol, triglyceride, high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) were measured at the clinical laboratory of Peking Union Medical College Hospital.

### Analyses and quantification of AAA

Four- to 6-month-old male mice fed a normal chow diet were used. All the mice were infused with AngII (Sigma-Aldrich, A9525, USA) at 1.44 mg kg<sup>-1</sup> day<sup>-1</sup> or saline for 4 weeks by using Alzet osmotic pumps (model 2004, DURECT Corp., USA). The experiments were performed as previously described (Satoh et al., 2009). An aortic aneurysm is defined as the increase in the external width of the suprarenal aorta by 50% or greater compared with that in saline-infused mice. The maximum width of the abdominal aorta was analyzed with Image Pro Plus software (Media Cybernetics).

### BAI treatment

One week before AngII or saline infusion, mice were pre-treated with BAI (Sigma-Aldrich, 465119, USA) dissolved in propylene glycol (vehicle). BAI was administered through intraperitoneal injection at two tentative dosages: 30 and 100 mg kg<sup>-1</sup> day<sup>-1</sup>. Majority of the mice in the 100 mg kg<sup>-1</sup> day<sup>-1</sup> group died of peritoneal inflammation within 1 week after BAI treatment. In contrast, all the mice in the 30 mg kg<sup>-1</sup> day<sup>-1</sup> group were in good condition during BAI treatment. Therefore, we selected 30 mg kg<sup>-1</sup> day<sup>-1</sup> dose for subsequent experiments. During AngII or saline infusion, experimental mice were also treated with the same dosage of BAI at the same time-point daily.

### Histological analyses

After hemodynamic measurements, the animals were sacrificed. For morphological analysis, the aortas were perfused with normal saline and fixed with 10% phosphate buffer saline (PBS) and formalin at physiological pressure for 5 min. The whole aortas were harvested, fixed for 24 h, embedded in paraffin, and cross-sections (5  $\mu$ m) were prepared. Paraffin sections were stained with elastin van Gieson, H&E, or used for immunostaining.

### Immunohistochemistry

Slides were deparaffinized, and endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide in 10% PBS for 10 min. Nonspecific binding sites were blocked with 10% bovine serum in PBS at room temperature for 1 h. Slides were incubated at 4°C overnight with diluted primary antibodies, then with biotinylated secondary antibody at 37°C for 30 min, and subsequently with horseradish peroxidase-labeled streptavidin solution for 20 min at 37°C. Slides were then stained with diaminobenzidine and counterstained with hematoxylin. The primary antibody used was CD45 (clone Ly-5, 1:100 dilution; BD Pharmingen, USA). A mean value was determined from at least 4 sections in each animal.

### Western blot analyses

Western blots were performed as described previously (Zhou et al., 2011). The primary antibodies used were mouse MMP-2 (sc-13595, 1:1000 dilution; SANTA CRUZ, USA), mouse GAPDH (5174, 1:3000 dilution; Cell Signaling Technology, USA), mouse MMP-9 (G657, 1:1000 dilution; Cell Signaling Technology, USA), mouse AT1R (sc-579, 1:2000 dilution; SANTA CRUZ, USA), mouse AT2R (sc-9040, 1:2000 dilution; SANTA CRUZ, USA), p38-MAPK (9212, 1:2000 dilution; Cell Signaling Technology, USA), phospho-p38-MAPK (9211, 1:2000 dilution; Cell Signaling Technology, USA), Erk1/2 (9102, 1:2000 dilution; Cell Signaling Technology, USA), phospho-Erk1/2 (9101, 1:2000 dilution; Cell Signaling Tech-



nology, USA), JNK (9252, 1:2000 dilution; Cell Signaling Technology, USA), and phospho-JNK (9251, 1:2000 dilution; Cell Signaling Technology, USA). Western blots were quantified densitometrically with Quantity One software (Bio-Rad), and the intensity values were normalized to GAPDH.

### Matrix metalloproteinase (MMP) activity

The detection of MMP-2 and MMP-9 activity was performed as previously reported (Hawkes et al., 2010). Five micrograms of protein in the concentrated aortic homogenates was electrophoresed on SDS-PAGE gels containing gelatin. Gels were washed in 2.5% Triton X-100 for 30 min and incubated for 12–40 h with zymography development buffer at 37°C. Subsequently, the gel was stained with Coomassie brilliant blue.

### ROS analysis

The aortic tissue was harvested, and the suprarenal abdominal aorta were embedded in OCT (C1400, APPLIED GEN, Beijing) and snap-frozen. DHE (5  $\mu\text{mol L}^{-1}$ , D-23107, Molecular Probes, USA) was topically applied to the freshly cut, frozen aortic sections (10  $\mu\text{m}$ ) for 30 min at 37°C to detect the presence of ROS as red fluorescence (585 nm) by confocal microscopy (Olympus).

### Statistical analyses

Quantitative results are expressed as the mean $\pm$ SE. Fisher's exact test was applied to the comparisons of AAA incidence and mortality. Comparisons of parameters between two groups were performed using the unpaired Student *t*-test or a nonparametric test. Comparisons of parameters between more than two groups were performed by one-way ANOVA, and comparisons of different parameters between each group were made by a post hoc analysis using the Bonferroni test. Statistical significance was evaluated with GraphPad Prism 5. A *P* value less than 0.05 was considered statistically significant.

**Compliance and ethics** The author(s) declare that they have no conflict of interest.

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